

REMARKS

Claims 8 and 10-15 are pending.

Claim Rejection under 35 U.S.C §103

Claims 8, 10-12, and 15 were rejected as obvious over Phillips (Plant Molecular Biology, vol. 24, pp. 603-615, 1994) in view of Wigler (U.S. Patent 5,436,142) and Frazer et al (Journal of Immunological methods, vol. 207, P 1-12, 1997) and further in view of Pinyopusarerk (ACIAR Proceedings, No. 16, pp. 147-148, 1987) was maintained. Claim 13 was rejected as obvious over Phillips in view of Wigler and Frazer et al and further in view of Pinyopusarerk as applied to claims 8, 10-12 and 15 above, and further in view of Nainan (J. Virol. Methods, vol. 61, pp. 127-134, 1996). Applicants respectfully traverse the rejections.

Phillips disclosed a method of cloning two gibberellin-regulated (GA-regulated) cDNAs from a plant, *Arabidopsis thaliana* (see title). Phillips' method includes a method of constructing enriched cDNA libraries by subtractive hybridization (see the legend of Fig. 1, p. 605). **The aim of the study of Phillips was to test the hypothesis that gibberellins induces stem elongation and flower development in *Arabidopsis thaliana* by changing gene expression** (see the first 3 sentences of the Abstract of Phillips).

In the subtractive hybridization method used by Phillips, Phillips took mRNA from a plant, *Arabidopsis thaliana*, treated with GA and made single stranded cDNA from the mRNA, wherein the single stranded cDNA had dG added at the 3' end (see the first 3

steps in Fig. 1). The single stranded cDNA of the GA-treated plant was subjected to subtractive hybridization with biotinylated mRNA (called the driver mRNA) from control *Arabidopsis thaliana* (not treated with GA) (see the 4th-6th steps in Fig. 1). The single stranded cDNA of the GA-treated plant that hybridized with the biotinylated mRNA of the control plant were removed by streptavidin (see the 5th step in Fig. 1, the first full paragraph on the right column in p. 606). The cDNA that remained after the subtractive hybridization contained DNA sequences that were regulated by GA (see the heading of the first paragraph under Results, p. 608). The cDNA that remained after the subtractive hybridization were used as templates to make double stranded cDNA followed by PCR amplification to form enriched cDNA libraries (see the last 2 steps in Fig. 1).

There are at least 6 differences between Phillips and the method of claim 8 described below. Applicants submit that the secondary and tertiary prior art references relied upon in the Office Action failed to rectify all of the differences between Phillips and claim 8 as discussed below.

First Difference:

Regarding step b) of claim 8, Phillips did not obtain genomic DNAs from two plant individuals. Instead, the first 3 steps in Fig. 1 of Phillips obtained only cDNA from one plant individual (the GA-treated plant).

Second Difference:

Regarding step c) of claim 8, the Office Action asserted that Phillips taught steps a-c of claim 8. Applicants respectfully disagree. Actually, Phillips did not subtract the genomic DNA of one plant individual from the genomic DNA of the other plant individual. Instead, the first 6 steps in Fig. 1 of Phillips can be interpreted to be equivalent to the subtraction of the cDNAs from two plant individuals (equivalent to the cDNA of GA-treated plant subtracted from the cDNA of the control plant).

The Office Action relied on Wigler in an attempt to cure the first and second differences (related to subtraction of genome DNA versus subtraction of cDNA) discussed above. But the attempt failed as explained below.

Column 2, lines 28-34, of Wigler disclosed that representational difference analysis, RDA, can be performed with genome DNAs as the DNA sources. Wigler also disclosed that the comparison in RDA can determine whether two related sources of DNA share a particular coding sequence (column 2, lines 58-63), but Wigler was silent on whether to use genome DNA or cDNA in the RDA to determine whether the two related sources of DNA share a particular coding sequence. Simply because Wigler taught that RDA can be performed using genome DNAs as the DNA sources, the Office Action regarded the first and second differences to be obvious. Applicants respectfully disagree.

Applicants contend that it would not have been obvious to modify the method of Phillips by using genome DNA, instead of cDNA, in the inter-individual DNA subtraction because **the aim of Phillips** was to study whether gibberellins induces phenotypic changes in the Arabidopsis plant **by changing gene expression**. In determining whether gibberellins changes the expression of genes, Phillips would not have been

interested in knowing whether there were any differences between the genome of the Arabidopsis plant treated with gibberellins and the genome of the Arabidopsis plant treated with water. In order to determine whether gibberellins caused any change in gene expression in Arabidopsis plants by following the teaching of Phillips, a person of ordinary skill in the art naturally would use cDNA in the subtraction and the person would have no motivation to compare genome DNAs, in place of cDNAs, from the Arabidopsis plant treated with gibberellins and the Arabidopsis plant treated with water. In other words, there would not have been any motivation to use the teaching of Wigler on using genome DNA in RDA to modify the method of Phillips. So the first and second differences of Phillips were not cured by Wigler. Since Frazer, Pinyopusarerk and Nainan did not teach subtraction of genome DNA from two individual plants, Frazer, Pinyopusarerk and Nainan also failed to cure the first and second differences of Phillips.

Third Difference:

Regarding step d), after the subtraction (via subtractive hybridization) conducted in the first 6 steps in Fig. 1, Phillips did not provide labeled cDNA probes derived from all mRNA obtained from the two plant individuals. The Office Action erroneously asserted that it would have been obvious to provide labeled cDNA probes from all mRNAs of the two plant individuals because Phillips provided single stranded cDNA from either the GA-treated plant or the control plant and because it would have saved time to provide the labeled cDNA probes from all mRNAs of the GA-treated plant and control plant (see page 4, lines 6-10, Office Action).

However, when Phillips talked about providing single stranded cDNA from either the GA-treated plant or the control plant (page 605, left column, the first 4 lines of the first full paragraph), Phillips was referring to the generation of the single stranded cDNA in the first 3 steps in Fig. 1, i.e. Phillips provided the single stranded cDNA from GA-treated plants to be used in the subtractive hybridization procedure between the two plant individuals. But step d) of claim 8 is not concerned with the subtractive hybridization between the two plant individuals (instead the method of claim 8 uses step c) to do DNA subtraction between two plant individuals). Thus, the Office Action's reasoning was applied to the wrong step of the method of claim 8 (i.e. the Office Action erroneously tried to cure a deficiency of Phillips related to step d) of claim 8 by relying on a teaching of Phillips that did not apply to step d)). Applicants respectfully contend that the obviousness rejection should be withdrawn because the Office Action failed to reasonably explain how the cited prior art could cure the third difference related to step d).

Fourth Difference:

Regarding step e), Phillips did not fractionate any DNA fragments obtained by inter-individual genome DNA subtraction and screen these DNA fragments with labeled cDNA probe derived from all mRNAs obtained from the two plant individuals. The Office Action erroneously asserted that Phillips taught step e) (see page 4, lines 12-13, Office Action). Applicants respectfully disagree.

Actually, Phillips did not fractionate any DNA fragments obtained by genome DNA subtraction. Instead, Phillips fractionated a small aliquot of the DNA fragments

obtained by cDNA subtraction using agarose gel electrophoresis in order to estimate the yield and size (see page 607, left column, lines 8-10), not in order to be screened with labeled cDNA probes derived from all mRNAs from the two plant individuals. The fourth difference is another reason why the obviousness rejection should be withdrawn.

Furthermore, Phillips did not teach screening DNA fragments obtained from inter-individual DNA subtraction with single stranded cDNA probes derived from all mRNAs obtained from the two plant individuals being compared. The Office Action erroneously asserted that Phillips taught step e) on the assertion that Phillips taught differential hybridization with labeled single stranded cDNA probes derived from mRNA from **GA-treated and control plants** (see the 3rd-6th lines from the bottom of page 4, Office Action). This assertion of the Office Action was incorrect because the differential hybridization done by Phillips was not applied to fractionated DNA fragments obtained from inter-individual genome DNA subtraction as required in step e) of claim 8. Instead, Phillips applied the differential hybridization to denatured DNA obtained by lysis of cells transfected with clones containing GA-regulated DNA sequences (see p. 607, left column, the last paragraph, especially lines 1-3 and 11-14; there was no differential hybridization of fractionated DNA fragments obtained from inter-individual DNA subtraction). This assertion of the Office Action was also wrong because Phillips in page 607, left column, did not use labeled cDNA derived from **GA-treated and control plants** in the differential hybridization. Instead, the differential hybridization was done by Phillips using labeled cDNA derived from **GA-treated or control plants** (see page 607, left column, last full paragraph, the 10th line from the bottom).

None of the other cited references, i.e. Wigler (which taught representational difference analysis, RDA), Frazer (which reviewed RDA), Pinyopusarerk (which described a program to improve the qualities of an Acacia tree) and Nainan (which disclosed nested PCR) taught step e) of claim 8, i.e. fractionating DNA fragments obtained by genome DNA subtraction and screening the DNA fragments with labeled cDNA probes derived from all mRNA of the two individuals being compared. Thus, the cited secondary and tertiary prior art references did not cure the fourth difference (i.e. related to step e) of claim 8) concerning Phillips.

For instance, even though Wigler taught that RDA may be used to compare two related sources of DNA to determine whether the two related sources of DNA **share** a particular sequence in the coding regions of the DNAs (column 2, lines 58-63), Wigler did not teach screening fractionated DNA fragments obtained from inter-individual genome DNA subtraction with single-stranded labeled cDNA probes derived from **all mRNAs obtained from the two individuals** under comparison. Wigler was silent on how to determine whether the particular coding sequence was shared by the two related sources of DNA. A DNA coding sequence **shared** by the two related sources of DNA will not be among the DNA fragments resulting from the inter-individual genome DNA subtraction. Even if a person of ordinary skill in the art were to use the method of Wigler to determine whether a particular coding sequence of interest is shared by two related sources of DNA, the person may screen the DNA fragments resulting from the inter-individual genome DNA subtraction, the person would have used a labeled cDNA probe derived from the mRNA for the particular coding sequence of interest obtained from one of the two individuals under comparison. But the person **would not** have

used labeled single stranded cDNA probes derived from all mRNAs obtained from the two individual under comparison as required by step e) of claim 8.

Even if the person of ordinary skill in the art were to follow Wigler's teachings to determine whether a particular **coding** sequence is shared by two related sources of DNA, the person would have used cDNAs in RDA (i.e. the person would have done inter-individual subtraction using cDNAs from the two individuals under comparison) instead of using genome DNAs in the RDA because using cDNAs would give stronger signals than using genome DNAs.

Fifth Difference:

Regarding step f) of claim 8, Phillips did not took DNA fragments identified by RNA-derived cDNA probes of the two plant individuals and performed intra-individual subtraction with genomic DNA from one of the plant individuals. Instead, Phillips used the GA-regulated DNA sequences cloned in the plasmid to construct a cDNA library (see the second full paragraph, right column, p. 607).

The Office Action attempted to rely on Frazer to cure the fifth difference. The Office Action asserted that Frazer taught adding a step of intra-individual DNA subtraction in RDA as a control step (page 8, right column, the first paragraph), so that a person of ordinary skill in the art would have modified the method of Phillips in view of Wigler by adding intra-individual DNA subtraction as a control step to account for any differences seen in inter-individual genome DNA subtraction (see page 11, Office Action). Applicants respectfully disagree.

Applicants contend that the reliance on Frazer (for the desirability of adding the intra-individual DNA subtraction as a control step in RDA) is misplaced due to two reasons. First, although Frazer stated that "Chang et al. (1994) have successfully taken this approach with genomic DNA" (page 8, right column, lines 15-16), actually Chang et al (a copy of which is attached) did not find intra-individual subtraction of genomic DNA to be very useful. Using RDA, Chang et al compared the genome DNA from two tissues, a diseased tissue and a normal tissue, from the same AIDS patient (page 1865, right column, lines 9-13). After "intra-individual" subtraction (i.e. intra-tissue subtraction) of genomic DNA using genomic DNA from one of the two tissues being compared in the "inter-individual" subtraction (i.e. inter-tissue subtraction), Chang et al found one DNA fragment, KS480Bam (page 1865, right column, the 6th to 9th lines from the bottom of the first full paragraph). However, with Southern hybridization, Chang et al found that two DNA fragments, KS480Bam and KS390Bam, nonspecifically hybridized to the tissues and were thus concluded to be artifacts (page 1865, right column, the last 6 lines). In other words, the "intra-individual" subtraction performed by Chang et al did not eliminate all unwanted DNA fragments from inter-individual subtraction. As a result, Chang et al did not support the desirability of doing intra-individual subtraction in RDA using genomic DNA.

Second, Frazer stated that "in our experience with RDA we have never isolated any housekeeping genes or other messages known to be present in both tester and driver populations originally" (page 8, right column, lines 17-20). Thus, Frazer taught that no intra-individual subtraction needs to be done as a control step in RDA. So Frazer taught away from the method of claim 8 in regarding to the fifth difference.

Thus, not only the reference (Chang et al) relied upon by Frazer did not support the desirability of doing intra-individual subtraction in RDA using genomic DNA, Frazer even **taught away from** doing intra-individual subtraction as a control step in RDA.

Sixth Difference:

Regarding step g), Phillips did not compare the DNA fragments obtained from genomic DNA inter-individual subtraction with the DNA fragments obtained from genomic DNA intra-individual subtraction of one of the plant individuals to exclude DNA fragments containing intra-individual polymorphisms in order to identify the DNA fragments that are polymorphic between the two plant individuals. Wigler and Pinyopusarerk were silent on intra-individual DNA subtraction, so Wigler and Pinyopusarerk could not cure the sixth difference between Phillips and claim 8. Although Frazer talked about intra-individual DNA subtraction in page 8, the right column, a person of ordinary skill in the art would not find any desirable reason from Frazer (because Frazer taught that no intra-individual subtraction needs to be done as a control step in RDA) of doing intra-individual DNA subtraction and excluding DNA fragments obtained from intra-individual subtraction from DNA fragments obtained from the inter-individual subtraction as required by step g) of claim 8.

The cited prior art did not suggest any modification of the method of Phillips by removing all 6 differences to arrive at the method of claim 8. Because the secondary and tertiary references cited in the Office Action failed to cure all 6 differences between Phillips and claim 8, the obviousness rejection should be withdrawn. The above arguments regarding claim 8 are also applicable to other rejected claims, so applicants

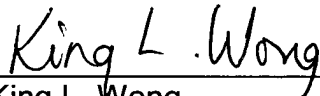
contend that all the claims should not have been rejected as obvious over the prior art cited.

Conclusion

In view of the above reasoning, applicants submit that the application is in a condition for allowance. A Notice of Allowance is believed in order.

In the event that the filing of this paper is not deemed timely, applicants petition for an appropriate extension of time. Any petition fee for the extension of time and any other fees that may be required in relation to this paper can be charged to Deposit Account No. 01-2300, referencing Docket No. 100021-09042.

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Attachment: Chang et al., *Science*, vol. 266, pp. 1865-1869, 1994

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REPORTS

(1989); M. D. Summers and G. E. Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures* (Texas Agricultural Station, College Station, TX, 1987). Deletions of SHC indicated in Fig. 2C were obtained by PCR and cloned into the same vector. GST-SHC fusion proteins were purified by binding to glutathione-agarose [K. Guan and J. E. Dixon, *Anal. Biochem.* 192, 282 (1991)]. The bound proteins were then incubated in 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 100 mM NaCl, 12 mM MgCl₂, 0.5 MCl of [³²P]ATP (5000 Ci/mmol), and 250 units of cAMP-dependent protein kinase catalytic subunit from ovine heart tissue for 1 hour at room temperature. The beads were then washed extensively and eluted with 10 mM glutathione. The specific activity of all preparations was typically >1 × 10⁷ cpm/μg. SDS-PAGE analysis showed a single band at the predicted size for the GST-SHC fusion proteins with other Coomassie staining or autoradiography. Immunoprecipitates or portions of cell lysates containing equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose. The filters were blocked for 2 hours at 4°C in nonfat dry milk (5%) in hybridization buffer [2.0 mM Hepes (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1 mM DTT, and 0.05% Triton X-100]. The filters were then incubated overnight at 4°C in hybridization buffer containing milk (1%), dried, and exposed to x-ray film with an intensifying screen for 6 to 36 hours at -70°C.

14. Lysate was prepared in hybridization buffer from

2.5 × 10⁷ BAL17 B cells stimulated by cross-linking the B cell antigen receptor as described [T. M. Sexton et al., *J. Immunol.* 153, 623 (1994)]. The lysate was incubated with approximately 250 ng of GST-SHCΔSH2 protein containing the IHA epitope (ag for 1 hour at 4°C). The mixture was then subjected to immunostaffin chromatography with the use of a monoclonal antibody to IHA covalently linked to agarose beads. The column was washed with 50 column volumes of hybridization buffer and eluted with 2% SDS. Proteins in equal fractions of the starting mixture, column flowthrough, and SDS eluate were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with ³²P-labeled PTB domain protein probe. In B cells, pp145 was seen as a doublet.

15. Anti-SHC immunoprecipitates from PDGF-stimulated fibroblasts immobilized on nitrocellulose filters were incubated in 25 mM imidazole (pH 7.0), 50 mM NaCl, 2.5 mM EDTA, 5 mM DTT, acetylated bovine serum albumin (100 μg/ml), and 5 units each of LAP and T cell tyrosine-specific phosphatases for 60 min at 30°C. An equivalent sample was treated identically except that 5 mM sodium orthovanadate was included. The filters were then washed extensively and blotted with ³²P-GST-SHC as above, except that the hybridization buffer included 1 mM sodium orthovanadate.

16. We thank P. P. Di Fiore and B. Knudsen for the Eps 15 and C3G antibodies, respectively, and W. J. Pantl, J. A. Escobedo, D. Schneider, and T. Quinn for reviewing the manuscript. Supported by NIH grants K11 HL02714 and F01 HL32898 and by the Doherty Research Center.

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Identification of Herpesvirus-Like DNA Sequences in AIDS-Associated Kaposi's Sarcoma

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Representational difference analysis was used to isolate unique sequences present in more than 90 percent of Kaposi's sarcoma (KS) tissues obtained from patients with acquired immunodeficiency syndrome (AIDS). These sequences were not present in tissue DNA from non-AIDS patients, but were present in 15 percent of non-KS tissue DNA samples from AIDS patients. The sequences are homologous to, but distinct from, capsid and tegument protein genes of the Gammaherpesvirinae, herpesvirus saimiri and Epstein-Barr virus. These KS-associated herpesvirus-like (KSHV) sequences appear to define a new human herpesvirus.

Kaposi's sarcoma is the most common neoplasm occurring in persons with AIDS; approximately 15 to 20% of AIDS patients develop this neoplasm, which rarely occurs in immunocompetent individuals (1). Epidemiologic evidence indicates that AIDS-associated KS (AIDS-KS) may have an infectious etiology. Gay and bisexual male AIDS patients are approximately 20 times more likely than hemophilic AIDS patients to develop KS, and KS may be associated with specific sexual practices among gay men with AIDS (2). KS is uncommon among adult AIDS patients infected through heterosexual or parenteral human immunodeficiency virus

(HIV) transmission, or among pediatric AIDS patients infected through vertical HIV transmission (3). Agents suspected of causing KS include cytomegalovirus (CMV), hepatitis B virus, human herpesvirus 6 (HHV6), HIV, and *Mycoplasma penetrans* (4). Extensive investigations, however, have not demonstrated an etiologic association between any of these agents and AIDS-KS (5). Noninfectious environmental agents, such as nitrite inhalants, also have been proposed to play a role in KS tumorigenesis (6).

To search for foreign DNA sequences belonging to an infectious agent in AIDS-KS, we used representational difference

analysis (RDA) to identify and characterize unique DNA sequences in KS tissue that are either absent or present in low copy number in nondiseased tissue obtained from the same patient (7). This method can detect adenovirus genome added in single copy to human DNA, but has not been used to identify previously uncultured infectious agents. RDA is performed by making simplified "representations" of genomes from diseased and normal tissues obtained from the same individual through polymerase chain reaction (PCR) amplification of short restriction fragments. The DNA representation from the diseased tissue is then ligated to a priming sequence and hybridized to an excess of unligated, normal-tissue DNA representation (8). Only unique sequences found in the diseased tissue that have priming sequences on both DNA strands are preferentially amplified during subsequent rounds of PCR amplification. This process can be repeated with different ligated priming sequences to enrich the sample for unique DNA sequences that are found only in the tissue of interest.

The initial round of amplification-hybridization from KS and excess normal-tissue DNA resulted in a diffuse banding pattern (Fig. 1, lane 2), but four bands at approximately 380, 450, 540, and 680 base pairs (bp) were identifiable after the second amplification-hybridization (Fig. 1, lane 3). These bands became discrete after a third round of amplification-hybridization (Fig. 1, lane 4). Control RDA, performed by hybridizing DNA extracted from AIDS-KS tissue against itself, produced a single band at ~540 bp (Fig. 1, lane 5). The four KS-associated bands (designated KS330Bam, KS390Bam, KS480Bam, and KS631Bam after digestion of the two flanking 28-bp ligated priming sequences with Bam HI) were gel purified.

KS390Bam and KS480Bam Southern (DNA) hybridized nonspecifically to both KS and non-KS human tissues and were not further characterized. The remaining two RDA bands, KS330Bam and KS631Bam, were cloned and sequenced (9). KS330Bam

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is a 330-bp sequence with a 51% G:C content (Fig. 2A), and KS631Bam is a 631-bp sequence with a 63% G:C content (Fig. 2B).

Both KS330Bam and KS631Bam code for amino acid sequences with homology to herpesviral polypeptides (10). KS330Bam

is 51% identical by amino acid homology to a portion of the ORF26 open reading frame encoding the capsid protein VP23 of herpesvirus saimiri (11), a gammaherpesvirus that causes fulminant lymphoma in New World monkeys. This fragment is also 39% identical to the amino acid sequence encoded by the corresponding BDLF1 ORF of Epstein-Barr virus (EBV) (12). The amino acid sequence encoded by KS631Bam has homology to the tegument protein (ORF75) of herpesvirus saimiri and to the tegument protein of EBV (ORF BNRF1, p140). KS631Bam is not significantly homologous to corresponding sequences of other herpesviruses.

Regions adjacent to KS330Bam were cloned and sequenced from a KS-tissue

Fig. 1. Agarose gel electrophoresis of RDA products from AIDS-KS tissue and nondiseased tissue. RDA was performed on DNA extracted from KS skin tissue and unaffected normal skin tissue obtained at autopsy from a homosexual man with AIDS-KS (8). Lane 1 shows the initial PCR-amplified genomic representation of the AIDS-KS DNA after Bam HI digestion. Lanes 2 to 4 show that subsequent cycles of ligation, amplification, hybridization, and digestion of the RDA products resulted in amplification of discrete bands at 380, 450, 540, and 680 bp. RDA of the extracted AIDS-KS DNA performed against itself resulted in a single band at 540 bp (lane 5). Bands at 380 bp and 680 bp correspond to KS330Bam and KS631Bam, respectively, after removal of 28-bp priming sequences. Bands at 450 and 540 bp [KS330Bam and KS480Bam, respectively] hybridized nonspecifically to both KS and non-KS human DNA. Lane M is a molecular size marker.

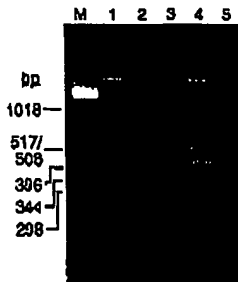


Fig. 2. Nucleotide sequences of the 1853-bp flanking region that includes KS330Bam derived from a KS lesion genomic library (A) and the KS631Bam sequence derived from KS tissue by RDA (B). KS330Bam (A) is underlined and Bam HI restriction sites (GGATCC) are double-underlined. A reading frame composed of the first 807 nucleotides (bp 1 to 807, stop codon in bold) is homologous to the COOH-termini of the major capsid protein open reading frames ORF25 of herpesvirus saimiri and BDLF1 of Epstein-Barr virus (EBV). An open reading frame from bp 633 to 1550 is homologous to ORF26 gene of herpesvirus saimiri and BDLF1 gene of EBV (start methionine codon (bp 633) and stop codon (bp 1548) in bold). A Pvu II site at bp 1086 (bold) marks the junction between 1- and 3-kb fragments cloned from the KS genomic library. The primer set for KS330₂₆ (bp 987 to 1008 and bp 1200 to 1219) and the internal probe used to detect the PCR amplification product (bp 1078 to 1102) are italicized.

A	10	20	30	40	50	60	70	80	90	100
KS330Bam	AGCTGAGAG	ATGATTCGGA	CCGCGTAC	ATCTGAGT	TTATATAC	AGACCCGAG	CAAGTCCGAG	CGTGTGAG	CGTGTGAG	GTGTGTGAT
	110	120	130	140	150	160	170	180	190	200
	CGTTCAGTA	AGGAAAGGCT	AGGATTTTTC	TTCTACAGC	ATTCAGACT	AGAGLCCGC	TACAGATGCC	CGTGTGAG	CAAGTCCGAG	CGTGTGAG
	210	220	230	240	250	260	270	280	290	300
	GTGTGTGAT	CCGCGTAC	CTATGAGTA	TTAGTTCG	CGACACGCT	CTGCGTAC	TTATATAC	TTGTGTGAT	TTGTGTGAT	TTGTGTGAT
	310	320	330	340	350	360	370	380	390	400
	TATGAGTAC	AATAGAGG	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	410	420	430	440	450	460	470	480	490	500
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	510	520	530	540	550	560	570	580	590	600
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	610	620	630	640	650	660	670	680	690	700
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	710	720	730	740	750	760	770	780	790	800
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	810	820	830	840	850	860	870	880	890	900
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	910	920	930	940	950	960	970	980	990	1000
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1010	1020	1030	1040	1050	1060	1070	1080	1090	1100
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1110	1120	1130	1140	1150	1160	1170	1180	1190	1200
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1310	1320	1330	1340	1350	1360	1370	1380	1390	1400
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1610	1620	1630	1640	1650	1660	1670	1680	1690	1700
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1810	1820	1830	1840	1850	1860	1870	1880	1890	1900
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	1910	1920	1930	1940	1950	1960	1970	1980	1990	2000
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT

B	10	20	30	40	50	60
KS631Bam	AGCTGAGAG	ATGATTCGGA	CCGCGTAC	ATCTGAGT	TTATATAC	AGACCCGAG
	70	80	90	100	110	120
	CGTTCAGTA	AGGAAAGGCT	AGGATTTTTC	TTCTACAGC	ATTCAGACT	AGAGLCCGC
	130	140	150	160	170	180
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	190	200	210	220	230	240
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	250	260	270	280	290	300
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	310	320	330	340	350	360
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	370	380	390	400	410	420
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	430	440	450	460	470	480
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	490	500	510	520	530	540
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	550	560	570	580	590	600
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT
	610	620	630	640	650	660
	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT	TTATGAGT

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DNA genomic library prepared from a single patient (13). This extended the contiguous sequence flanking both sides of KS330Bam to 1853 bp (Fig. 2A). A com-

plete open reading frame at bp 633 to 1550, which included the KS330Bam sequence, was confirmed to be homologous to the ORF26 and BDLF1 open reading

frames (55% and 56% matching nucleotide identity, respectively) of herpesvirus saimiri and EBV (11, 12). Significantly lower homologies exist to corresponding proteins of bovine herpesvirus type 4, HHV6, CMV, and human herpesvirus 7 (HHV7).

The polypeptide encoded by the KS-associated DNA open reading frame shows extensive amino acid homology to the proteins encoded by herpesvirus saimiri ORF26 and EBV BDLF1 (Fig. 3). Although it is homologous to these herpesvirus regions, the polypeptide does not match any other known sequence and thus provides evidence for a viral genome related to but distinct from known members of the herpesvirus family. In addition, the 5' end of the 1853-bp sequence (bp 1 to 607) is 66% and 67% identical to corresponding regions of the major capsid protein (MCP) genes of herpesvirus saimiri (ORF25) and EBV (BcLF1), respectively. In both EBV and herpesvirus saimiri genomes, the MCP gene is found immediately adjacent to the BDLF1-ORF26 gene (11, 12). This region also has lower degrees of similarity to MCP genes of other human herpesviruses, including HSV1, VZV, HHV6, CMV, and HHV2 (14).

To determine the specificity of KS330Bam and KS631Bam for AIDS-KS, these sequences were random-primed, ³²P-labeled, and hybridized to Southern blots of DNA extracted from cryopreserved tissues obtained from patients with and without AIDS (15). Twenty of 27 (74%) AIDS-KS DNA specimens hybridized with variable intensity to both KS330Bam and KS631Bam, and one additional KS specimen hybridized only to KS631Bam by Southern blotting (Fig. 4 and Table 1). In contrast to AIDS-KS lesions, only 6 of 39 (15%) non-KS tissues from patients with AIDS hybridized to KS330Bam and KS631Bam. Specific hybridization did not occur with lymphoma or lymph node DNA from 36 persons without AIDS or with control DNA from 49 tissue biopsy specimens obtained from a consecutive series of patients. DNA specimens extracted from vascular tumors and tissues with opportunistic infections common in AIDS were also negative (Table 1). In addition, DNA samples from EBV-infected peripheral blood lymphocytes and pure cultures of *Mycobacterium avium*-complex were negative as well. Overall, 20 of 27 (74%) AIDS-KS specimens hybridized to KS330Bam and 21 of 27 (78%) AIDS-KS specimens hybridized to KS631Bam, as compared to only 6 of 142 (4%) non-KS human DNA control specimens ($\chi^2 = 85.02$, $P < 10^{-7}$ and $\chi^2 = 92.4$, $P < 10^{-7}$, respectively).

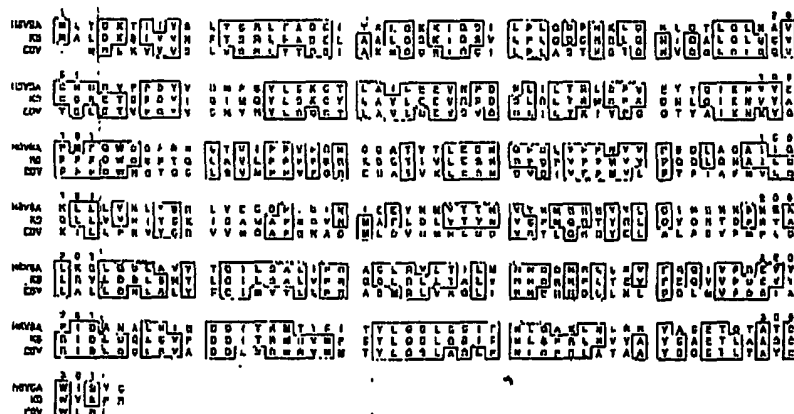


Fig. 3. Comparison of protein sequences encoded by ORF26 from herpesvirus saimiri (HVS), and BDLF1 from EBV, to the protein encoded by the KS-associated DNA open reading frame. Regions of amino acid identity between KS and HVS, or KS and EBV are outlined (24).

Table 1. Southern blot hybridization for KS330Bam and KS831Bam and by PCR amplification for KS330₂₀₀ in human tissues from individual patients.

Tissue type	n	No. positive by KS330Bam DNA hybridization (%)	No. positive by KS631Bam DNA hybridization (%)	No. positive by KS330 ₂₃₃ PCR (%)
AIDS-KS	27*	20 (74)	21 (78)	25 (83)
AIDS lymphomas	27†	3 (11)	3 (11)	3 (11)
AIDS lymph nodes	12	3 (25)	3 (25)	3 (25)
Non-AIDS lymphomas	28‡	0 (0)	0 (0)	0 (0)
Non-AIDS lymph nodes	7	0 (0)	0 (0)	0 (0)
Vascular tumors	5§	0 (0)	0 (0)	0 (0)
Opportunistic infections	13	0 (0)	0 (0)	0 (0)
Consecutive surgical biopsies	49¶#	0 (0)	0 (0)	0 (0)

*Includes one AIDS-KS specimen unsuitable for p53 exon 6 and one tissue which on microscopic examination did not have any detectable KS tissue present. Both of these samples were negative by Southern blot hybridization to KS330Bam and KS831Bam and by PCR amplification for the KS330₂₃₃ amplicon. Comparison of AIDS-KS KS330Bam, KS831Bam, and KS330₂₃₃ results to each of the control tissue subgroups is significant ($P < 0.01$). 1-tail Fisher's exact test (FET). For comparisons between AIDS-KS and AIDS lymphomas, the odds ratios and 1-TET P-values for KS330Bam, KS831Bam, and KS330₂₃₃ positivity were: 22.0, $P = 3 \times 10^{-6}$; 28, $P = 8 \times 10^{-7}$; and 100, $P < 10^{-7}$, respectively. For comparisons between AIDS-KS and AIDS lymph nodes, the odds ratios and FET P values for KS330Bam, KS831Bam, and KS330₂₃₃ positivity were: 8.0, $P = 0.006$; 10.5, $P = 0.004$; and 38, $P = 4.7 \times 10^{-6}$, respectively. Includes 7 small nonfollicular-cell lymphomas, and 20 diffuse large-cell and immunoblastic lymphomas. Three of the lymphomas with immunoblastic morphology were positive for KS330Bam and KS831Bam. Includes 13 anaplastic large-cell lymphomas, 4 diffuse large-cell lymphomas, 4 small lymphocytic lymphomas-chronic lymphocytic leukemias, 3 hairy-cell leukemias, 2 monoclonal B-cell lymphomas, 1 follicular small cleaved-cell lymphoma, 1 Burkitt's lymphoma, and 1 plasmacytoma. Includes 2 angiosarcomas, 1 hemangioendothelioma, 1 lymph node with vascular transformation, and 1 lymphangioma. Includes 2 cryptococci, 1 toxoplasmosis, 1 cat-scratch bacillus, 1 CMV, 1 EBV, and 7 acid-fast bacillus-infected tissues. In addition, pure cultures of *Mycobacterium avium*-complex were negative by Southern hybridization and PCR, and pure cultures of *Mycobacterium penetrans* and lymphocyte cultures with EBV were negative by PCR (not included). (Tissues included: skin, appendix, kidney, prostate, hernia sac, lung, fibrous tissue, gallbladder, colon, torsekin, thyroid, anal bowel, adenoid, vein, axillary tissue, lipoma, heart, oral mucosa, hemorrhoid, pseudotumor, and testis track. Tissues were collected from a consecutive series of biopsies on patients without AIDS but with unknown HIV serostatus. #Apparent nonspecific hybridization at approximately 20 Kb occurred in four consecutive surgical biopsy DNA samples: one colon and one hernia sac DNA sample hybridized to KS330Bam alone, another hernia sac DNA sample hybridized to KS831Bam alone, and one appendix DNA sample hybridized to both KS330Bam and KS831Bam. These samples did not hybridize in the 330- to 630-bp range expected for these sequences and were PCR negative for KS330₂₃₃.

The sequence copy number in the AIDS-KS tissues was estimated by simultaneous and a hybridization with KS330Bam 440-bp probe for the single-copy conserved region of the T cell receptor β gene (16). Samples in lanes 5 and 6 of Fig. 4 showed similar intensities for the two probes, indicating an average copy number of approximately two KS330Bam sequences per cell, whereas remaining KS tissues had weaker hybridization signals for the KS330Bam probe.

These results were confirmed and extended by PCR amplification with primers designed from KS330Bam (Fig. 2A) that amplify a 233-bp subfragment (17) designated KS330₂₃₃. Although Southern blot hybridization detected the KS330Bam sequence in only 20 of 27 KS tissues, 25 of the 27 tissues were positive by PCR amplification for KS330₂₃₃ (Fig. 5A), demonstrating that KS330Bam is present in some KS lesions at levels below the threshold for detection by Southern blot hybridization. The two AIDS-KS specimens that were negative for KS330₂₃₃ ap-

peared to be so for technical reasons: One had no microscopically detectable KS tissue in the frozen sample (Fig. 5A, lane 3), and the other (Fig. 5A, lane 15) was negative in the control PCR amplification for the p53 gene (18), indicating either DNA degradation or the presence of PCR inhibitors in the sample. All KS330₂₃₃ PCR products hybridized to a ³²P end-labeled 25-bp internal oligonucleotide, confirming the specificity of the PCR (Fig. 5B).

Except for the six non-KS control samples from AIDS patients that were positive by Southern blot hybridization, none of the other 136 non-KS control specimens were positive by PCR for KS330₂₃₃. Overall, DNA samples from 25 (93%) of 27 AIDS-KS tissues were positive by PCR, as compared to 6 (15%) of 39 non-KS lymph nodes and lymphomas from AIDS patients ($\chi^2 = 38.2$, $P < 10^{-6}$), 0 of 36 lymph nodes and lymphomas from non-AIDS patients ($\chi^2 = 55.2$, $P < 10^{-7}$), and 0 of 49 consecutive biopsy specimens ($\chi^2 = 67.7$, $P < 10^{-7}$). All control specimens were amplifiable for p53, indicating that inad-

equated PCR amplification was not the reason for lack of detection of KS330₂₃₃ in the control tissues. Thus, KS330₂₃₃ was found in all 25 amplifiable tissues with microscopically detectable AIDS-KS, but rarely occurred in non-KS tissues, including tissues from AIDS patients. Additional DNA samples from EBV-infected lymphocytes and from *M. penetrans* (ATCC #55252), a candidate KS agent (19), were negative for KS330₂₃₃. Several KS-tissue DNA samples tested with EBV-specific and mycoplasma-specific consensus PCR primers were also negative (20).

Of the six control tissues from AIDS patients that were positive by both PCR and Southern hybridization, two patients had KS at other sites, two did not develop KS, and complete clinical histories for the remaining two patients were unobtainable. Three of these tissues were lymph nodes with follicular hyperplasia taken from patients with AIDS. Undetected microscopic KS foci may have been present in these lymph nodes, given the high lifetime occurrence of KS (>50%) in some

Table 2. Differential detection of KS330Bam, KS631Bam, and KS330₂₃₃ sequences in KS-affected (KS) and unaffected autopsy tissues from four patients with AIDS-KS. Patients A, B, and C were gay males with AIDS and patient D was a female intravenous drug user with AIDS.

Tissue type	KS330Bam	KS631Bam	KS330 ₂₃₃
Patient A			
KS, skin	+	+	+
Skin	+	+	+
Muscle	+	+	+
Patient B			
KS, skin	+	+	+
Muscle	-	-	-
Brain	-	-	-
Patient C			
KS, stomach	+	+	+
Stomach, adjacent to KS	-	-	-
Muscle	-	-	-
Brain	-	-	-
Colon	-	-	-
Heart	-	-	-
Hilar lymph nodes	-	-	-
Patient D			
KS, skin	+	+	+
Skin, adjacent to KS	-	-	+
Hilar lymph node	-	-	+
Mesenteric lymph node	-	-	-
Brain	-	-	-
Lung	-	-	-
Stomach	-	-	-
Spleen	-	-	-
Liver	-	-	-
Muscle	-	-	-

Fig. 4. Hybridization of ³²P-labeled KS330Bam (A) and KS631Bam (B) sequences obtained by RDA to a representative panel of 19 DNA samples extracted from KS lesions and digested with Bam HI. KS330Bam hybridized to 11 of the 19 and KS631Bam hybridized to 12 of 19 DNA samples from the AIDS-KS lesions shown. Two cases (lanes 12 and 13) showed faint bands with both KS330Bam and KS631Bam probes after longer exposure. One negative specimen (lane 3) did not have microscopically detectable KS in the tissue specimen. Seven of 8 additional KS DNA samples not shown also hybridized to both sequences.

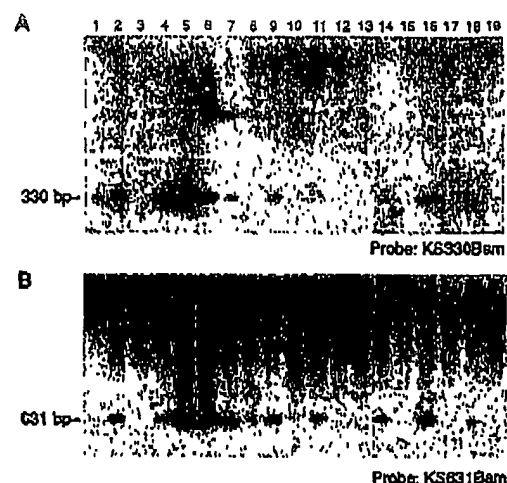
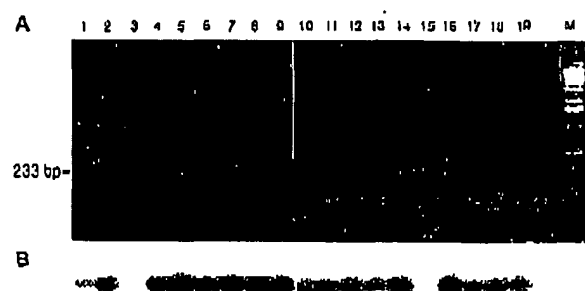


Fig. 5. PCR amplification of the 19 KS-derived DNA samples shown in Fig. 4, using the KS330₂₃₃ primers shown in Fig. 2. (A) shows the agarose gel of the amplification products from 19 KS DNA samples (lanes 1 to 19), and (B) shows specific hybridization of the PCR products to a ³²P end-labeled 25-bp internal oligonucleotide (Fig. 2) after transfer of the gel to a nitrocellulose filter. Negative samples in lanes 3 and 15, respectively, lacked microscopically detectable KS in the sample or did not amplify the human p53 exon 8, suggesting that these samples were negative for technical reasons. An additional eight AIDS-KS samples were amplified and all were positive for KS330₂₃₃. Lane 20 is a negative control and lane M molecular size marker.



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risk groups of AIDS patients (21). Alternatively, these lymph nodes may have been asymptomatically infected with, or may have been incubating, the putative agent. The other three positive tissue specimens were a form of B cell immunoblastic lymphoma from AIDS patients. Given the previously noted association between KS and lymphoproliferative disorders (22), it is possible that the putative KS agent is also a cofactor for a subset of AIDS-associated lymphomas. A comparison of AIDS-KS tissues to only lymph node and lymphoma tissues from AIDS patients demonstrates that KS330Bam and KS631Bam remain significantly associated with the KS phenotype when controlling for concurrent AIDS, indicating that HIV disease is not a confounding factor in our analysis. Among only AIDS tissue samples from separate patients (Table 1), over 90% of KS specimens (100% of confirmed and amplifiable KS specimens) were positive for KS330₂₃₃, as compared to 15% of lymph node and lymphoma tissues from AIDS patients. These sequences therefore appear to be specifically associated with KS in AIDS patients, although it is not clear whether the presence of these sequences is causal or is an epiphenomenon of KS.

To show that KS330Bam and KS631Bam are not heritable polymorphic DNA markers for KS, we tested multiple unaffected tissue DNA samples from four additional patients with AIDS-KS (Table 2). Whereas KS lesion DNA samples were positive by Southern hybridization and PCR, unaffected tissues were generally negative for these sequences. All other tissues except muscle and unaffected skin from patient A, stomach adjacent to the KS lesion in patient C, and adjacent skin and hilar lymph nodes in patient D were negative. These results are consistent with an infectious process and may represent local and disseminated spread of the putative virus.

Although these sequences suggest the presence of a new human herpesvirus in KS lesions, a causal link between these sequences and AIDS-KS cannot be established by our retrospective case control study. It is possible that this agent is a common latent virus in humans that preferentially colonizes KS lesions in immunosuppressed patients. Unlike previous studies searching for agents associated with KS, the sequences found in our study were present in all intact KS DNA samples from a large number of patients and were preferentially found in diseased as compared to normal tissues from the same host. Our results have been independently confirmed with 100% concordance in a blinded PCR evaluation with extracted

AIDS-KS lesion DNA and non-KS brain DNA from the same patients (23).

REFERENCES AND NOTES

1. V. Boral, T. A. Perleman, R. L. Burkholder, H. W. Jaffe, *Lancet* 335, 123 (1990); V. Boral et al., *Br Med J* 302, 624 (1991).
2. V. Boral et al., *Lancet* 339, C32 (1992); C. P. Archibald et al., *Epidemiology* 3, 203 (1992); A. R. Lison et al., *Am. J. Epidemiol.* 131, 221 (1990); M. T. Schecter et al., *ibid.* 134, 485 (1991).
3. T. A. Perleman, H. W. Jaffe, A. E. Friedman-Kien, R. A. Weiss, in *Cancer Survivors*, vol. 10, *Cancer, HIV and AIDS* (Imperial Cancer Research Fund, London, 1991), pp. 23-27.
4. W. L. Drew et al., *Lancet* 335, 125 (1990); A. Siddiqui et al., *Proc. Natl. Acad. Sci. U.S.A.* 80, 4881 (1983); P. Bovero et al., *Lancet* 341, 1288 (1993); J. Vogel et al., *Nature* 333, 606 (1990); R. Y.-H. Wang et al., *Clin. Infect. Dis.* 17, 724 (1993); G. Grillo, E. Beth, F. M. Buonaguro, *Antibiot. Chemother.* 32, 1 (1983).
5. F. van den Berg et al., *J. Clin. Pathol.* 42, 128 (1989); G. S. Johnston, J. Jockusch, L. C. McMurty, W. X. Shender, *Cancer Detect. Prev.* 14, 337 (1989); N. Jahan et al., *AIDS Res. Hum. Retroviruses* 5, 225 (1989); S. D. Hornberg, *Cancer Detect. Prev.* 14, 351 (1989).
6. H. W. Haverkos, F. F. Pinskey, P. Drotman, D. J. Bregman, *Sex. Transm. Dis.* 12, 203 (1985).
7. N. Laitayn, N. Laitayn, M. Wiegler, *Source* 259, 946 (1993).
8. DNA specimens (10 µg) extracted from both the KS lesion and unaffected tissue were separately digested to completion with Bam HI (20 U/µg) at 37°C for 2 hours, and 2 µg of digestion fragments were ligated to NBam12 and NBam24 priming sequences (primer sequences described in (7)). Thirty cycles of PCR amplification were performed to amplify "representations" of both genomes. After construction of the genomic representations, fragments of DNA between 100 and 1500 bp (Fig. 1, lane 1) were isolated from an agarose gel, and NBam priming sequences were removed by digestion with Bam HI. To search for unique DNA sequences not found in non-KS DNA, a second set of priming sequences (JBam12 and JBam24) was ligated onto only the KS fragments (Fig. 1, lane 1). The ligated KS DNA fragments (0.2 µg) were hybridized to 20 µg of unligated fragments representing normal lesion DNA. A sample of the hybridization product was then subjected to 10 cycles of PCR amplification with JBam24, followed by mung bean nuclease digestion. A sample of the mung bean-treated difference product was then subjected to 15 more cycles of PCR with the JBam24 primer (Fig. 1, lane 2). Amplification products were redigested with Bam HI, and 200 ng of the digested product was ligated to RBam12 and RBam24 primer sets for a second round of hybridization and PCR amplification (Fig. 1, lane 3). This enrichment procedure was repeated a third time with the JBam primer set (Fig. 1, lane 4). Both the original KS DNA and the DNA from non-KS tissue used in the RDA (Table 2, patient A) were subsequently found to contain the AIDS-KS-specific sequences KS330Bam and KS631Bam, indicating that RDA can be successfully used when the target sequences are present in unequal copy number in both tissues.
9. Gel-purified RDA products were cloned in the pCRII vector through use of the TA cloning system (Invitrogen, San Diego, CA). Sequencing was done with Sequencing version 2.0 (U.S. Biochemical) system according to the manufacturer's instructions. Nucleotide sequences were confirmed with an Applied Biosystems 373A Sequencer in the DNA Sequencing Facility at Columbia University.
10. SmartProt and PIR protein databases were searched for homologous ORF with BLASTX [S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* 215, 403 (1990)].
11. J.-C. Albrecht et al., *J. Virol.* 66, 5047 (1992).
12. R. Baer et al., *Nature* 310, 207 (1984).
13. KS330Bam was used as a probe to select eight cross-hybridizing λ phage clones. DNA from one of these clones was digested with Pvu II, which cuts once within KS330Bam (pp 1084, Fig. 2A) and probed with labeled KS330Bam DNA. Two hybridizing bands, ~1.1 kb and 3 kb in length from opposite sides of the Pvu II site, were identified and subcloned. The entire 1.1-kb fragment and 768 bp of the 3-kb fragment were sequenced for homology comparisons.
14. Use of BLASTX (10) for local alignment of the translated six-frame nucleotide sequence to the NCBI NR database resulted in the following list of herpesviridae MCP alignments, in decreasing order of homology (Poiseux probabilities and percentage amino acid identity of major HSP in parentheses): HVS (8.1 × 10⁻¹⁰⁰, 70%), EBV (2.0 × 10⁻⁸⁰, 67%), bovine herpesvirus type 4 (1.0 × 10⁻⁷³, 73%), HHV1 (5.0 × 10⁻⁵⁸, 40%), equine herpesvirus type 1 (1.9 × 10⁻⁵², 41%), VZV (2.0 × 10⁻⁴⁰, 40%), and herpesvirus type 1 (3.3 × 10⁻¹⁶, 61%), HHV8 (1.8 × 10⁻¹⁴, 25%), HHV7 (6.7 × 10⁻¹⁴, 23%), and CMV (3.5 × 10⁻¹², 27%).
15. The tissues, listed in Table 1, were collected from diagnostic biopsy and autopsies between 1983 and 1993 and stored at -70°C. Each tissue sample was from a different patient. Most of the 27 KS specimens were from lymph nodes dissected under surgical conditions, which diminishes possible contamination with normal skin flora. All AIDS-KS specimens were examined microscopically for morphologic confirmation of KS and immunohistochemically for latent HIV, Ulex europaeus, and CD34 antigen expression. One of the AIDS-KS specimens was apparently mislabeled, because KS tissue was not detected on microscopic examination but was included in the KS specimen group for purposes of statistical analysis. Additional clinical and demographic information on the specimens was not collected to preserve patient confidentiality.
16. P. G. Pellicci, D. M. Knowles, R. Dallo-Favara, *J. Exp. Med.* 162, 1015 (1985).
17. The conditions for PCR analyses were as follows: 94°C for 2 min (1 cycle); 94°C for 1 min, 58°C for 1 min, 72°C for 1 min (35 cycles); 72°C extension for 5 min (1 cycle). Each PCR reaction used 0.1 µg of genomic DNA, 50 pmol of each primer, 1 U of Taq polymerase, 100 µM of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), and 0.1% Triton X-100 in a final volume of 25 µl. Amplifications were carried out in a Perkin-Elmer 460 Thermocycler with 1-s ramp times between steps.
18. PCR amplification of the human p53 tumor suppressor gene was used as a control for DNA quality. Sequences of p53 primers derived from published sequences are as follows: P5-3', 5'-ACAGGCTGCTG-3' (TCCOCAGGT-3', P6-3', 5'-AGTTGCAACCA-GACCTCAG-3' [G. Geland et al., *Proc. Natl. Acad. Sci. U.S.A.* 88, 6413 (1991)]).
19. S.-C. Lo et al., *Int. J. Syst. Bacteriol.* 42, 357 (1992).
20. Mycoplasma PCR detection kit, Stratagene, La Jolla, CA; J. C. Lin, S. C. Lin, B. K. De, W. P. Chan, D. L. Evans, *Blood* 81, 3372 (1993).
21. M. H. Katz et al., *J. Infect. Dis.* 170, 198 (1994).
22. R. J. Biggar, R. E. Curtis, T. R. Cole, C. S. Rubkin, M. Melbye, *Am. J. Epidemiol.* 139, 362 (1994); N. Bendtsen, M. Dittler, J. Blomberg, S. Ågren, K. Mark, *Eur. J. Cancer* 28, 899 (1990); B. Satal, V. Miké, G. Giraldo, E. Beth, H. A. Good, *Cancer* 45, 1472 (1980).
23. L. Karpowicz, unpublished data.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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